Free radical activity of industrial fibers: role of iron in oxidative stress and activation of transcription factors

Free Radical Activity of Industrial Fibers: Role of Iron in Oxidative Stress and Activation of Transcription Factors

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We studied asbestos, vitreous fiber (MMVF10), and refractory ceramic fiber (RCF1) from the Thermal Insulation Manufacturers' Association fiber repository regarding the following: free radical damage to plasmid DNA, iron release, ability to deplete glutathione (GSH), and activate redox-sensitive transcription factors in macrophages. Asbestos had much more free radical activity than any of the man-made vitreous fibers. More Fe^{3+} was released than Fe^{2+} and more of both was released at pH 4.5 than at pH 7.2. Release of iron from the different fibers was generally not a good correlate of ability to cause free radical injury to the plasmid DNA. All fiber types caused some degree of oxidative stress, as revealed by depletion of intracellular GSH. Amosite asbestos upregulated nuclear binding of activator protein 1 transcription factor to a greater level than MMVF10 and RCF1; long-fiber amosite was the only fiber to enhance activation of the transcription factor nuclear factor κB (NFκB). The use of cysteine methyl ester and buthionine sulfoximine to modulate GSH suggested that GSH homeostasis was important in leading to activation of transcription factors. We conclude that the intrinsic free radical activity is the major determinant of transcription factor activation and therefore gene expression in alveolar macrophages. Although this was not related to iron release or ability to deplete macrophage GSH at 4 hr, GSH does play a role in activation of NFκB. — *Environ Health Perspect* 105(Suppl 5):1313–1317 (1997)

Key words: asbestos, fiber, MMVF, RCF, free radicals, oxidative stress, glutathione, transcription factors

Introduction

Extensive research in laboratory animals and human populations on the effects of asbestos has determined that exposure increases the risk of pulmonary fibrosis, pleural mesothelioma, and bronchial carcinoma (*1,2*). Much less is known of the potential toxicity of man-made respirable industrial fibers (*2*). Early research into the mechanisms of fiber pathogenicity implicated fiber geometry, length and dimension (*2,3*), and surface area (*4*). The physical properties of respirable fibers appear to persist in the lung milieu and express their pathogenic potential and their ability to cause genetic effects *in vitro* (*5*). Man-made vitreous fiber(s) (MMVF) and refractory ceramic mineral fibers (RCF) also have been shown to be pathogenic in animal inhalation studies (*6*). Surface-associated free radical activity has been suggested as a mechanism of asbestos-mediated toxicity (*7*), but man-made fibers appear to have little activity in this respect (*8*). Free radicals may cause damage by causing DNA, lipid, and protein oxidation but more subtle effects in initiating gene transcription may also occur (*9*). Iron is released readily from selected fibers (*8*) and can produce free radicals via Fenton chemistry, which ultimately results in the production of the hydroxyl radical.

In the present study we examine the free radical activity of three fibers and relate this to the release of iron and its ability to cause oxidative stress and to activate nuclear transcription factors AP-1 and NFκB in alveolar macrophages.

Materials and Methods

Fibers

For this study asbestos and two types of man-made fibers were used. The amosite asbestos was used as previously described (*10*). RCF 1, 2, 3, and 4, and MMVF 10, 11, 21, and 22 were obtained from the Thermal Insulation Manufacturers' Association (TIMA) fiber repository and were as used in the Research and Consulting Company (RCC) studies (*6*). The characteristics of these different samples are as previously described (*8*). All fibers were used at equal fiber numbers of 9.3×10^5 fibers/DNA assay, 8.24×10^7/ml for iron detection, and 8.24×10^6/ml for reduced glutathione (GSH) and gel mobility shift assays.

Measurement of Iron Release from Fibers

All fiber types were suspended in either pH 4.6 or pH 7.2 citric acid/$NaHPO_4$ buffer at a fiber concentration of 8.24×10^7 fibers/ml and iron release over 8 hr at 37°C was assessed using desferrioxamine-B (DSF-B) for Fe^{2+}, or ferrozine 10 mM for Fe^{3+} (*8*). The amount of iron present in solution after 8 hr of incubation was expressed as μM Fe^{2+} or $Fe^{3+}/8.24\times10^7$ fibers.

Measurement of Induction of Fiber-mediated Free Radical Damage to DNA

As described in detail by Gilmour et al. (*8*), φ X174 RF1 closed-circular supercoiled DNA was used to detect free radical activity.

Alveolar Macrophage Preparation

Alveolar macrophages were obtained by lavage from in-house-bred, adult Wistar rats, as described previously. Macrophages

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Abbreviations used: AP-1, activator protein 1; BSO, buthionine sulfoximine; CME, cysteine methyl ester; DSF-B, desferrioxamine-B; GSH, reduced glutathione; GSSG, oxidized glutathione; LFA, long-fiber amosite; LPS, lipopolysacharride; Man, mannitol; MMVF, man-made vitreous fiber(s); NFκB, nuclear factor κB; RCC, Research and Consulting Company; RCF, refractory ceramic fiber(s); TIMA, Thermal Insulation Manufacturers' Association.

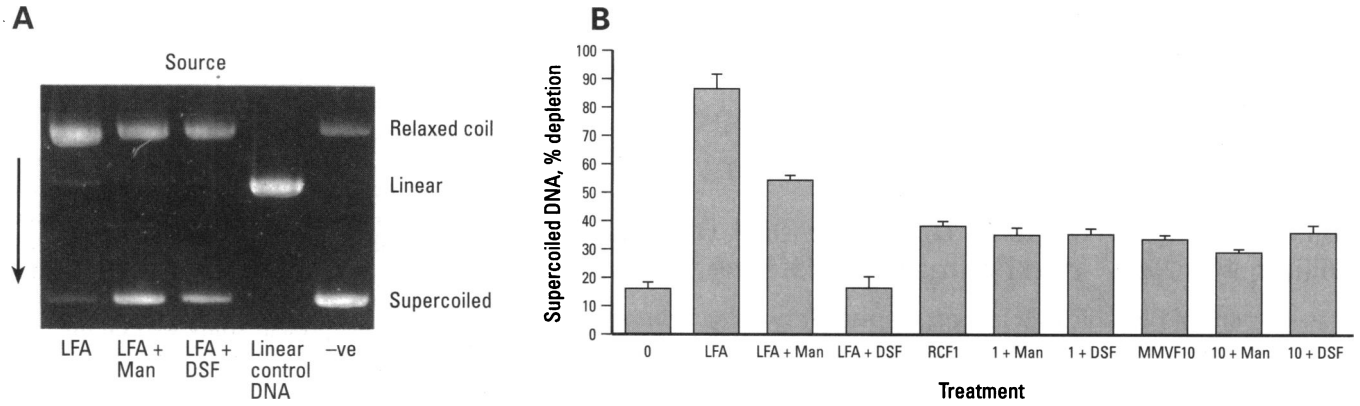

Figure 1. (*A*) Diagram of the supercoiled DNA plasmid electrophoresis gel indicating the free radical depletion of the supercoiled fraction into relaxed coil and linear plasmid forms. (*B*) Quantification of supercoiled DNA depletion by fiber treatments, demonstrating the role of hydroxyl radical and iron in radical damage to plasmid DNA. This figure and all subsequent figures are the mean and SEM of at least three separate experiments. Man, mannitol.

were seeded into 24-well plates at a concentration of 1×10^6 cells/ml and allowed to attach for 1 hr, after which nonadherent cells were removed by washing with medium and cell treatments were performed in the culture medium for 4 hr. For gel mobility shift assays 3×10^6 rat alveolar macrophages were seeded and incubated with treatment for 16 hr.

GSH and GSSG Assays

The alveolar macrophages were assessed for GSH and oxidized glutathione (GSSG) by spectrophotometry according to the modified method of Tietze (*11,12*). The protein content of the cell lysates was measured by the BioRad Bradford protein reagent BioRad Laboratories (Hemel, Hempstead, UK) to allow the GSH and GSSG to be described as nanomolar per milligram of protein. To alter the levels of GSH, cysteine methyl ester (CME) was incubated with macrophages for 30 min before fiber addition at a concentration of 300 µM to increase the GSH levels. Buthionine sulfoximine (BSO) was used similarly to decrease the GSH at a concentration of 100 µM.

Gel Mobility Shift Assays

Nuclear proteins were extracted from treated, washed macrophages, and all gel mobility shift assays were carried out according to the method modified by Nicholson et al. (*13*). Briefly, alveolar macrophages were treated with fibers for 4 hr, after which nuclear proteins were extracted as previously described (*14*). For each mobility shift reaction, 7 µg of nuclear protein was incubated with 1.75 pmol of freshly ^{32}P-labeled AP-1 or NFκB oligonucleotide. This was carried out at room temperature in the presence of 0.25 mg/ml poly(dI-dC)•poly(dI-dC) competitor oligonucleotides to prevent nonspecific nuclear protein–DNA binding. Samples were separated in 6% polyacrylamide gel for 18 hr at 20 V. Bands were quantified using scanning laser densitometry.

Statistical Analysis

One- or two-way analysis of variance was used to determine the significance of treatment effects. The Tukey correction was used when comparisons between multiple treatment groups were required.

Results

Fiber-mediated Free Radical Activity

Amosite asbestos produced considerable free radical damage to DNA, as shown by the significant 85% depletion compared to 10% for untreated controls ($p \leq 0.001$). This asbestos free radical activity was partially attributable to hydroxyl radicals, as shown by mannitol intervention and chelation of fiber-associated iron (Figure 1). RCF1 and MMVF10 fibers were significantly less able to produce free radicals in this assay ($p \leq 0.001$), and the slight reduction in supercoiled DNA attributed to these fibers could not be accounted for by iron or hydroxyl radical (Figure 1).

Release of Iron from Fibers

To assess a possible connection between free radical activity and fiber-associated iron and the effect of physiologically important pHs, the release of iron from a range of fibers was determined. More Fe^{3+} than Fe^{2+} was released at both pH 4.6 and 7.2; there clearly was more iron released at pH 4.6 than at 7.2 (Figure 2). There did not appear to be any correlation between iron release and free radical activity because both RCF1 and MMVF10 released substantial amounts of iron, with RCF1 releasing significantly more iron of both forms than long-fiber amosite (LFA) (Figure 2).

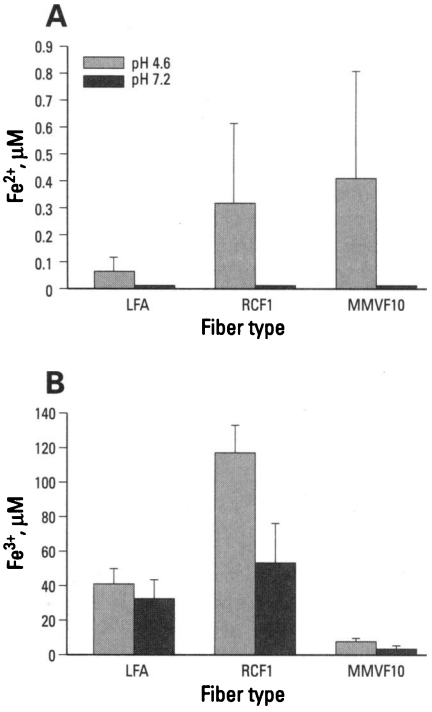

Figure 2. Release of (*A*) Fe^{2+} and (*B*) Fe^{3+} at pH 4.6 and 7.2 from fibers, demonstrating the increased release of Fe^{3+} compared to Fe^{2+} and greater release at pH 4.6.

Rat Alveolar Glutathione Concentration

All fibers significantly lowered intracellular GSH ($p \leq 0.01$), with MMVF10 causing

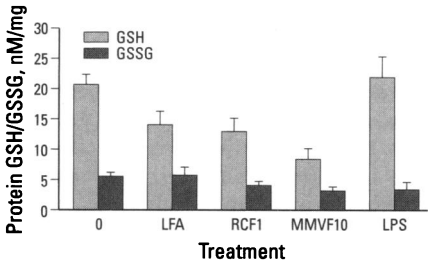

Figure 3. Levels of intracellular GSH and GSSG after treatment with different fibers.

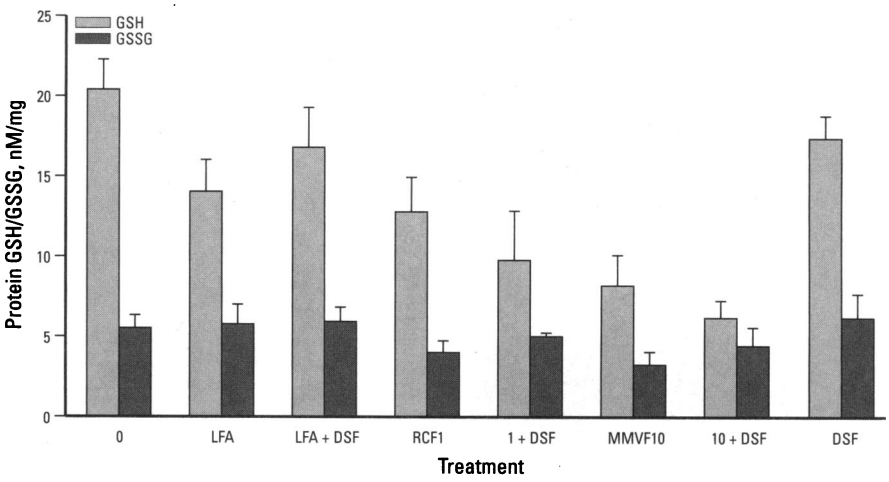

Figure 4. Levels of GSH and GSSG after treatment with normal fibers and fibers treated with the iron chelator DSF. Note no significant effect of chelation of fiber-associated iron.

the greatest depletion (Figure 3). Chelation of fiber-associated iron did not significantly ameliorate the fiber-mediated decrease in GSH levels, except in the case of LFA treatment, indicating that iron was not responsible for the intracellular redox status (Figure 4).

Transcription Factors

Compared to that in the untreated control, MMVF10 and RCF1 fiber samples upregulated the nuclear translocation of AP-1 transcription factor by 11.9 and 9.1% respectively. LFA, however, produced a far greater increase in AP-1, 37.4% more than in the untreated control. This was similar to the levels found with the lipopolysaccharide (LPS) positive control sample (Figure 5). Only LFA was able to upregulate the NFκB transcription factor; MMVF10 and RCF1 produced no effect (Figure 5).

To test the effect of lowering intracellular GSH on the fiber-mediated NFκB activation, fibers were incubated with GSH-depleted cells and NFκB activation was measured. Depletion of GSH increased the upregulation attributed to LFA treatment and also produced upregulation of RCF1 almost double that of the BSO alone control (Figure 6).

To further study the role of GSH in transcriptional activation, we supplemented the intracellular GSH with CME. In cells pretreated this way LFA-mediated NFκB nuclear translocation was almost eliminated.

Discussion

The substantial free radical activity associated with LFA, attributable largely to hydroxyl radicals, agrees with the results of our own and other previous studies (*7,8,15*). This reflects the pathogenicity of this form of asbestos; Johnston and Maples (*16*) have demonstrated that this free radical activity can induce p53 protein through iron via Fenton chemistry. LFA

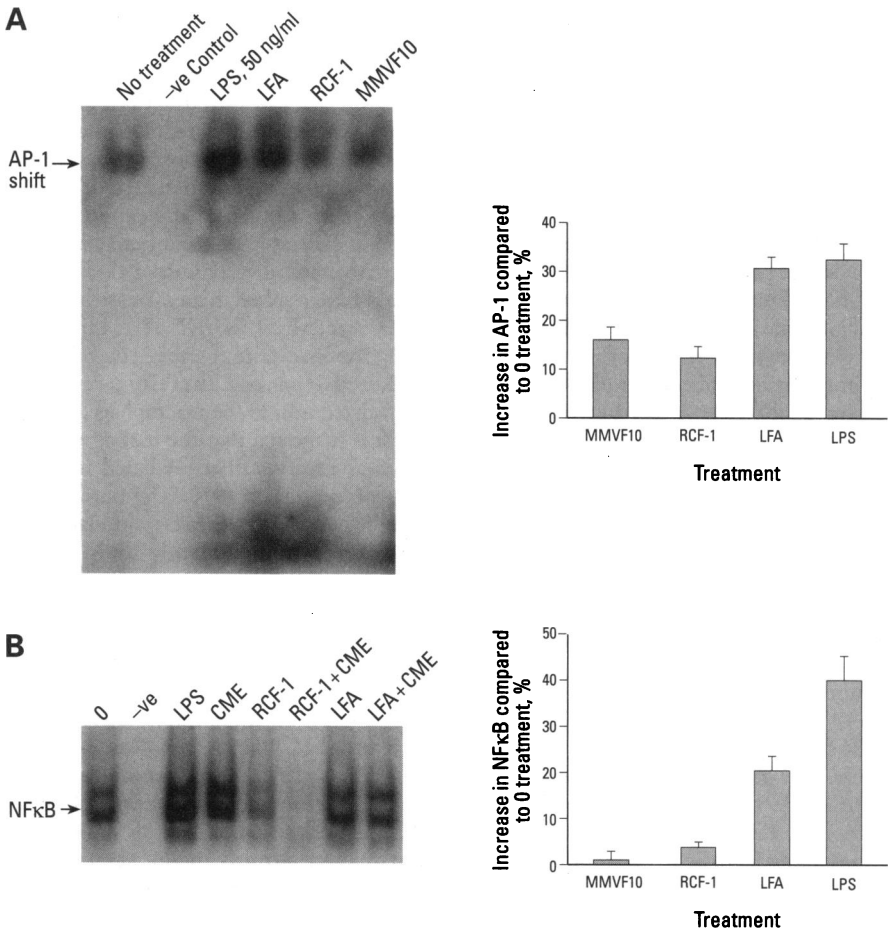

Figure 5. Nuclear translocation of (*A*) AP-1 and (*B*) NFκB transcription factors with fiber treatments and control. Graph shows quantification of bands; each bar represents the mean and ± SEM values from three separate experiments.

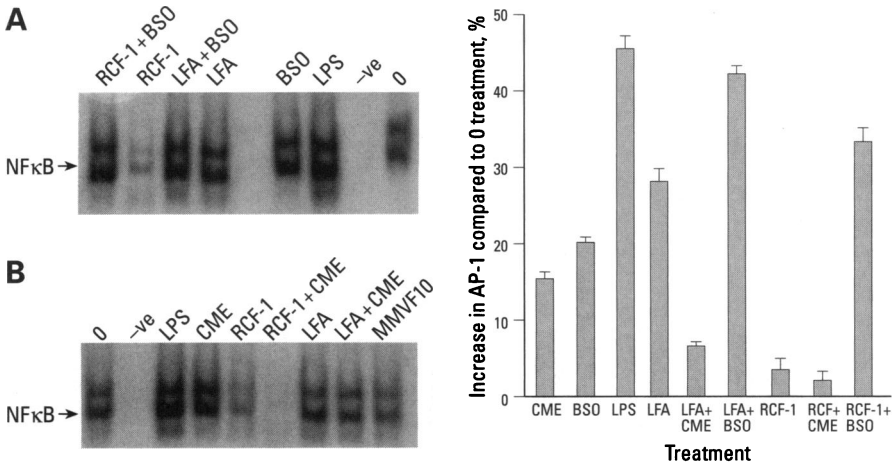

Figure 6. (*A*) Nuclear translocation of NFκB on fiber treatment for control cells and cells pretreated with BSO; LPS treatment as + control. (*B*) Nuclear translocation of NFκB on fiber treatment for control cells and cells pretreated with CME; LPS treatment as + control. Each bar represents the mean and ± SEM values from three separate experiments. Graph shows quantification of nuclear translocation of NFκB shown in the autoradiographs.

has been shown to have a large oxygen radical-generating potential compared to that of other fibers (*17*), and indeed this appeared to be the case in this study where MMVF10 and RCF1 displayed only minimal free radical activity. The minimal free radical generation displayed by MMVF10 and RCF1 could not be accounted for by iron or hydroxyl radicals. The lack of surface activity appears to agree with the relative lack of pathogenicity of MMVF10, but RCF1 fibers have been found to be carcinogenic in animal studies (*6*).

The extent of release of the two forms of iron from the fibers at both pHs did not relate to the extent of fiber free radical activity. Although iron was responsible for the activity of asbestos, as demonstrated by chelation studies, both MMV10 and RFC1 released comparable amounts of, if not more, iron. Therefore, the ability of fibers to generate free radicals cannot be accounted for by released iron alone. The importance of iron in free radical activity, however, may lie in the behavior of surface-bound iron; this is under further investigation. The presence in the lung milieu of reductants initiating Fenton chemistry could be a factor in the pathogenicity of man-made fibers that release iron, but obviously this is unimportant in the DNA assay. Phagocytosis by macrophages produces reductants that have been shown to enhance the production of dust-related free radicals (*18*) and all three fibers used in the present study caused significant depletions of macrophage GSH. The ability to cause GSH depletion is not, therefore, related to the free radical activities of the fibers. Thus, the products of phagocytosis are likely to mediate some of the oxidative stress effects of fibers. Iron chelation at the fiber surface did not affect the ability of fibers to change GSH redox status, which indicates that GS depletion depends on the presence of iron. The decrease in GSH is likely to be a result of the exportation of GSH as a stress response instead of a direct fiber/free radical oxidation of GSH, which would have resulted in an increase in GSSG. However, the low pH of macrophage phagolysosomes may cause the release of chelated iron and therefore re-expose unchelated iron to the macrophage.

It is suggested that the pathogenicity of asbestos involves fiber-mediated initiation of gene expression (*19*). The upregulation of NFκB and AP-1 transcription factors by LFA, but not significantly by MMVF10 and RCF1, indicates a relationship between fiber free radical activity and gene expression. However, although GSH depletion may play a role in the activation of these transcription factors in other situations (*20*), all three fibers caused depletion of GSH. Pharmacological depletion of GSH with BSO alone did increase upregulation of NFκB and enhanced RCF1-mediated upregulation, so redox status is important in the activation of transcription, as previously shown (*19*). Supplementing intracellular GSH with CME inhibited the LFA-mediated NFκB activation, again confirming that GSH status is important. The fact that all fibers lowered GSH but only LFA caused transcriptional activation, conflicts with the evidence that pharmacologic modulation of GSH did influence transcriptional activation; this may be explained at several levels. The time course of GSH depletion may be central to the activating effect and the 4-hr time point used here may not reflect the most important GSH change. The effect of direct fiber reactivity with cellular thiols may be important in this system in which the thiol redox status of the cell is important in thiol-dependent transcription factor activation. In addition to the effect of fibers on anti-oxidant thiols, there is the possibility that transcription factor-related thiols may be directly influenced by the fibers, masking the relative activation potentials of the fiber samples. These possibilities require further study to determine the mechanism of the activation cascade and to differentiate how the different properties of these fibers result in differing cell responses. Also involved may be other oxidative stress-sensitive molecules such as carbon-based free radical and other intracellular signaling pathways. These are under further investigation.

In conclusion, the hydroxyl radical activity of asbestos exceeds that of MMVF10 and RCF1 fibers and iron does not appear to be a determining factor in this free radical-generating potential or in their relative pathogenicity. The greater free radical activity of LFA was mirrored in the activation of NFκB and AP-1 transcription factors. All three fibers depleted GSH at the 4-hr time point used here. These results indicate that the free radical activity of asbestos and other fibers may be a key factor in to gene transcription in macrophages, but the exact role of GSH in these events remains to be elucidated.

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